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From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

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Office, PCT
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Applicant KIM, Byunghong et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

02 February 2001 (02.02.01)

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International Application No.

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Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* 2000-P-34

Box No. I TITLE OF INVENTION An Electrochemical Method for Enrichment of Microorganism, a Biosensor for Analyzing Organic Substance and BOD

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

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Sheet No. 2

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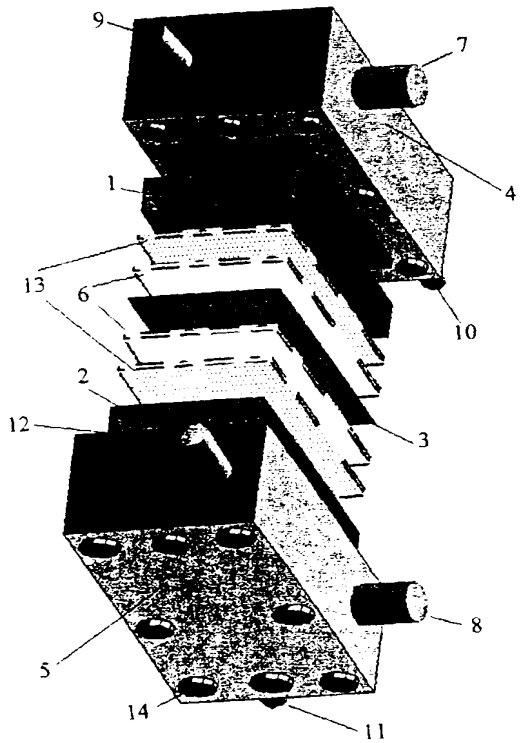
(54) Title: AN ELECTROCHEMICAL METHOD FOR ENRICHMENT OF MICROORGANISM, A BIOSENSOR FOR ANALYZING ORGANIC SUBSTANCE AND BOD



(57) Abstract: Disclosed herein is a biosensor that allows an organic substance concentration or BOD of a sample to be electrochemically measured in anaerobic condition using a mediator-less biofuel cell. The biosensor utilizes electrochemically active bacteria that were contained in wastewater and sluge and densely cultured during the operation procedure of the biofuel cell for the BOD measurement, as a microbial catalyst of the biofuel cell used in the biosensor. As a result, the biosensor can be operated without an artificial addition of microorganisms, and allows the microorganisms to be maintained at a suitable activity depending on the nature of wastewater. In addition, the biofuel cell used in the biosensor can be operated in a stable manner over six months or more.

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AN ELECTROCHEMICAL METHOD FOR ENRICHMENT OF MICROORGANISM, A BIOSENSOR FOR ANALYZING ORGANIC SUBSTANCE AND BOD

Technical Field

The present invention relates to a biosensor for the measurement of an organic substance concentration and BOD. More particularly, the present invention relates to a biosensor for measuring an organic substance concentration and BOD, which biosensor enables the performance of a simple and rapid measurement, and is relatively inexpensive in costs required for its, fabrication, application, maintenance, and repair.

Background Art

In general, a biosensor means a measuring device in which organisms or substances originated from the organisms are used for at least one part of a measuring unit that is coupled with an electrical device. The biosensor has been continuously studied from the 1960's, as it has an advantage in that it enables the precious measurement of concentration and properties of a substance to be measured, by virtue of a high degree of specificity with a biological reaction. As a result, a variety of biosensors were developed, and substances to be measured became varied in their range. For instance, there are put to practical use and widely used a glucose concentration-measuring biosensor fabricated of a glucose oxidase coupled to an oxygen electrode, and a medical biosensor containing an antibody (see, Tuner et al., 1987, Biosensors, Fundamentals and Applications, Oxford Science Publications).

Meanwhile, the pollution level of an industrial wastewater or a domestic sewage is generally represented in terms of Chemical Oxygen Demand (COD) or Biochemical Oxygen Demand (BOD). Their rapid measurements have a significantly important value in environmental and pollution prevention-allied industries. However, the prior method for measuring BOD that shows the amount of microorganism-magnetizable organic substances is problematic in that it requires much time, as well as various complex procedures and devices for the measurement.

Moreover, the prior method is disadvantageous in that a variation in measured value occurs depending on a skilled degree of workers. In addition, this method is difficult to apply to the case where the polluted state needs to be rapidly identified or where an automated facility for wastewater treatment is installed.

To solve these problems, there were proposed several kinds of biosensors for the measurement of BOD (see, Hikuma et al., 1979, European Journal of Microbiology and Biotechnology, 8, 289; Riedel et al., 1990, Water Research, 24, 883; and Hyun et al., 1993, Biotechnology and Bioengineering, 41, 1107). Generally, these BOD sensors have a structure in which a membrane, onto which a certain microorganism is immobilized, is attached to a dissolved oxygen-measuring electrode. Where these BOD sensors are reacted with a sample to be measured, the microorganism immobilized onto the membrane magnetizes an organic material contained in the sample while consuming oxygen. The value of dissolved oxygen in the resulting sample is compared to the value of dissolved oxygen in a control sample and converted into BOD. However, these biosensors have the following problems:

First, these biosensors employ one microorganism species. Thus, they are short of the magnetic susceptibility to complex nutrient components present in wastewater due to the substrate specificity of the used microorganism, thereby being not capable of indicating the total value of BOD.

Second, a microorganism is immobilized on a porous membrane. For this reason, the membrane needs to be frequently replaced or repaired in order for BOD to be measured at a high reproducibility. However, as the microorganism-immobilizing membrane is expensive, the biosensors are uneconomical, and also poor in maintainability.

Third, as there shall be used a dissolved oxygen electrode for a control sample, the equipment is complex, and also is high in equipment cost and failure rate.

Fourth, a microorganism used in these BOD-measuring biosensors can not transfer electrons directly to its outside. For this reason, the biosensors require the use of an electron transfer mediator or a separate transducer.

Meanwhile, microorganisms growing in an anaerobic environment can commonly utilize electron receptors other than oxygen. The metabolism using these electron receptors is named the anaerobic respiration of microorganisms. The electron receptors, which can be used in the oxidation of an organic substance by the anaerobically respiratory microorganisms, include ferric oxide, nitrate, hexavalent manganese, sulfate, carbonate and the like. If the electron donors are the same, the reduction of ferric oxide into ferrous oxide generates the largest level of energy among energy generated from the redox reactions between the respective electron receptors and the electron donor, with the energy level being low in order of nitrate, sulfate and carbonate. This energy level is associated with the redox potential which is an inherent characteristic of the respective electron receptors (see, Byoung-Hong, Kim, Microorganism Physiology, Academy Press Co., Ltd., Seoul, Korea, 1995).

Among these electron receptors used by the metal salt-reducing bacteria that anaerobically respire, ferrous oxide and the like are very low in solubility in water. This insoluble electron receptor can not be absorbed into, and reduced in microorganism cells, unlike oxygen that is a common electron receptor used by the aerobic microorganisms. Thus, in the metal salt-reducing bacteria, there is present a specific form of an electron transfer system in order to reduce the electron receptor present in the outside of the cells. For instance, in *Geobacter sulfurreducens* and *Shewanella putrefaciens* that are a kind of the metal salt-reducing bacteria using ferric oxide as an electron receptor, there is present cytochrome, an electron transfer protein. Through this cytochrome, electrons generated from the oxidation of organic substances within the microorganisms are transferred to the electron receptor outside of the microorganism cell. Using energy generated by this electron transfer procedure, the microorganism grows. [See, Myers and Myers, Journal of Bacteriology, 174, 3429-3438, (1992); and Seeliger et al., Journal of Bacteriology, 180, 3686-3691, (1998)]. As a result, these metal salt-reducing bacteria having similar characteristics transport electrons generated from a catabolism of organic substances to the external insoluble electron receptor such that the receptor is reduced. For this reason, the amount of the organic substances will be proportional to the amount of the reduced electron receptor. Also, when a

suitable electrode is used that can be substituted for the electron receptor, the electrode will be reduced with the electrons generated from the inside of the bacteria, and the electrons transferred directly to the electrode will outwardly flow through a circuit. A biofuel cell using such physiological characteristics of the microorganisms is described in Korean Patent Application Publication No. 1998-16777 (June 5, 1998), the disclosure of which is incorporated herein by reference.

In the biofuel cell including the use of the metal salt-reducing bacteria, the quantity of the generated electrons is in proportion to a concentration of the bacteria, the amount of the organic substances and the like. Thus, the measurement of the quantity of the generated electrons allows the amount of the organic substances present in the sample to be determined.

Accordingly, we have continued to study such biofuel cells, and microorganisms and organic substances that can be used in the biofuel cell. As a result of that, we have perfected the present invention.

Disclosure of the Invention

It is therefore an object of the present invention to provide an improved biosensor for the measurement of BOD, and a method for the measurement of BOD using the same, which biosensor has no drawbacks with the prior biosensors for the measurement of BOD.

In accordance with a first aspect of the present invention, there is provided a BOD-measuring biosensor, comprising a measuring unit, an electric current-detecting unit, and a recording unit serving to record a variation in the detected electric current, the measuring unit being composed of a mediator-less biofuel cell, the biofuel cell including: cathodic and anodic compartments defined therein and contained with a conductive medium, respectively; an anode arranged in the anodic compartment; a cathode arranged in the cathodic compartment; and an ion exchange membrane interposed between the cathodic and anodic compartments and serving to divide the anodic compartment from the cathodic compartment, wherein the anodic compartment is added with a sample containing electrochemically active bacteria.

According to a second aspect of the present invention, there is provided a method for measuring BOD of a sample using the BOD-measuring biosensor of the

first aspect above, the method comprising: electrically connecting the anode to the cathode via a resistor; introducing nitrogen into the anodic compartment to maintain the anodic compartment in an anaerobic condition, while introducing oxygen into the cathodic compartment to maintain the cathodic compartment in an aerobic condition; densely culturing electrochemically active bacteria present in the sample in the anodic compartment; and measuring electric current being generated while employing the densely cultured, electrochemically active bacteria as a microbial catalyst.

In a third aspect of the present invention, there is provided a mediator-less biofuel cell type biosensor for the measurement of organic substance concentration, the biosensor comprising a measuring unit, an electric current-detecting unit, and a recording unit serving to record a variation in the detected electric current, the measuring unit being composed of a mediator-less biofuel cell, the biofuel cell comprising: cathodic and anodic compartments defined therein and contained with a conductive medium, respectively; an anode arranged in the anodic compartment; a cathode arranged in the cathodic compartment; and an ion exchange membrane interposed between the cathodic and anodic compartments and serving to divide the anodic compartment from the cathodic compartment, wherein the anodic compartment contains a single species of electrochemically active bacterium serving to catabolize the organic substance.

In a fourth aspect of the present invention, there is provided a method for measuring a concentration of an organic substance using the biosensor according to the third aspect above, the method comprising: adding a sample to be measured to the anodic compartment while continuing to feed air to the cathodic compartment to maintain the cathodic compartment at a voltage different from the anodic compartment; and measuring an electric current generated from the consumption of an organic substance contained in the sample by the electrochemically active bacterium, whereby the concentration of the organic substance is measured.

In a fifth aspect of the present invention, there is provided a method for densely culturing electrochemically active bacteria present in active sludge and wastewater, using the mediator-less biofuel cell included in the biosensor according to the third aspect above, the method comprising: adding an active sludge and a

wastewater to the anodic compartment; electrically connecting the anodic compartment to the cathodic compartment via a resistor; introducing nitrogen to the anodic compartment to maintain the anodic compartment in an anaerobic condition, while introducing air to the cathodic compartment to maintain the cathodic compartment in an aerobic condition; whereby a bacterium present in the active sludge and wastewater is densely cultured without a separate electron receptor.

Brief Description of the Drawings

The above and other objects and aspects of the invention will be apparent from the following description of embodiments with reference to the accompanying drawings, in which:

Fig. 1 is a perspective view schematically showing a biofuel cell used in a BOD-measuring biosensor according to the present invention;

Fig. 2 is a graph showing a correlation of electric current with COD of a sample added to a biofuel cell according to Example 1 of the present invention;

Fig. 3 is a graph showing a correlation of the quantity of the generated electricity with COD of a sample added to a biofuel cell according to Example 1 of the present invention;

Fig. 4 is a schematical view showing a BOD-measuring biosensor according to Example 2 of the present invention, with the biosensor including the use of a microorganism dense-culturing device having a potentiostat;

Fig. 5 is a graph showing a correlation of electric current with COD of a sample added to a biofuel cell type biosensor in which an electrochemically active bacterium was densely cultured using a potentiostat according to Example 2 of the present invention;

Fig. 6a is a scanning electron micrograph of the surface of a working electrode of the biofuel cell type biosensor according to Example 2 of the present invention, which micrograph was taken before the biosensor is used for densely culturing microorganisms.

Fig. 6a is a scanning electron micrograph of the surface of a working electrode of the biofuel cell type biosensor according to Example 2 of the present

invention, which micrograph was taken after the biosensor is used for densely culturing microorganisms.

Fig. 7 is a schemetical view of a biofuel cell type biosensor for measuring a lactic acid concentration according to Example 4 of the present invention;

Fig. 8 shows typical increase in electric current generated during the measurement of a lactic acid concentration;

Fig. 9 is a graph showing a correlation of a lactic acid concentration with an initial slope of the generated electric current, which was obtained according to Example 4 of the present invention; and

Fig. 10 is a graph showing the quantity of electricity according to COD of a sample, which electricity was measured for six months using the BOD-measuring biosensor according to Example 1 of the present invention.

Best Mode for Carrying Out the Invention

The present invention is directed to a biosensor capable of measuring a concentration of microorganism-magnetizable components (BOD) or organic substances, such as lactic acid, that are present in wastewater. For such a measurement, the inventive biosensor employs an organic substance-magnetizing force and electron transfer capacity of electrochemically active microorganisms without an electron transfer mediator or a transducer.

In one embodiment of the present invention, the BOD-measuring biosensor comprises a measuring unit, an electric current-detecting unit, and a recording unit serving to record a variation in the detected current. The measuring unit is composed of a mediator-less biofuel cell. Such a biofuel cell includes cathodic and anodic compartments defined therein and contained with a conductive medium, respectively. Further, the biofuel cell includes an anode arranged in the anodic compartment; a cathode arranged in the cathodic compartment; and an ion exchange membrane interposed between the cathodic and anodic compartments and serving to divide the anodic compartment from the cathodic compartment. In the anodic compartment, there is included a sample containing an electrochemically active bacteria.

More specifically, in the anodic compartment, the electrochemically active bacteria are electrochemically densely cultured using, as a seed sample, organic substances and active sludge present in a certain sample. The densely cultured, electrochemically active bacteria is used as a microbial catalyst to produce electric power. The produced electric power is proportional to a concentration of various organic substances, which are magnetizable by microorganisms added to the biofuel cell that serves as the measuring unit. Thus, the detection and recording of the produced electric power allow BOD of the sample to be determined.

Moreover, in order to facilitate the dense culture of the electrochemically active bacteria in the anodic compartment of the measuring unit, there may be preferably used a potentiostat.

As used herein, the term "electrochemically active bacteria" means bacteria that can discharge electrons generated from the oxidation of an organic substance present in wastewater to the outside of their cells to transfer the electrons to an electrode, thereby generating electric current. An example of the electrochemically active bacteria typically includes metal salt-reducing bacteria.

In another embodiment of the present invention, a biosensor for the measurement of an organic substance concentration contains electrochemically active bacteria at its electrode itself or electrode compartment. Such bacteria utilize certain organic substances as a substrate. The biosensor containing such bacteria is used by itself as the measuring unit. That is to say, as the anodic compartment contains the electrochemically active bacteria of catabolizing a certain organic substance, electric power generated by the biofuel cell corresponds to that generated by the catabolism of certain organic substances present in a sample. Thus, the measurement of the generated electric power allows a concentration of the organic substances present in the sample to be determined.

The method for measuring BOD and an organic substance concentration as described above will now be described in detail.

(1) Dense Culture of Electrochemically Active Bacterium, and Biosensor for Measuring BOD Using the Cultured Bacterium

From recent studies, it was confirmed that active and anaerobic sludges originated from wastewater have a variety of metal salt-reducing bacteria including a

large amount of iron-reducing bacteria in a high concentration [see, Nielsen et al., Systematic and Applied Microbiology, 20, 645-651, (1997); Nielsen et al., Water Science and Technology, 34, 129-136, (1996); and Rasmussens et al., Water Research, 28, 417-425, (1994)].

Accordingly, if a seed sample, in which various species of microorganisms are mixed with each other, is anaerobically cultured along with a suitable culture in a fermenter including electrodes, only microorganisms that can use the electrode as an electron receptor are then finally viable. These microorganism species have an electron carrier such as cytochrome, and thus have an electrochemical activity. As a result, in accordance with such a manner, it is possible to selectively densely culture microbes having an electrochemical activity among various species of microorganisms present in wastewater and active sludge.

Meanwhile, as wastewater and contaminated sewage contain various organic substances, it is very difficult to measure BOD of wastewater or sewage in a uniform manner, i.e., using only one species of microorganism. In addition, this measurement is high in errors. For these reasons, according to the present invention, various species of electrochemically active bacteria present in organic wastewater and active sludge are densely cultured as described above, and the densely cultured active bacteria are used as a microbial catalyst of the biofuel cell of the measuring unit, thereby generating electric power. From the quantity of the produced electric power, BOD of the sample can be determined.

(2) Measurement of an Organic Substance Concentration Using Biofuel Cell Type Biosensor

The anodic compartment of the above described biofuel cell in the BOD-measuring biosensor is added with a single species of an electrochemically active microorganism selected depending on the nature of a substrate to be measured. The cathodic compartment is continued to feed air such that it is maintained at a voltage level different from the anodic compartment. The anodic compartment is added with the sample to be measured. Then, the microorganisms contained in the anodic compartment consume the corresponding substrate, while electrons being produced flow out to an external circuit through the anodic compartment. The measurement of the produced electric current allows a concentration of the

corresponding substrate to be determined. In this way, the use of the electrochemically active bacteria consuming various substrates allows a concentration of the corresponding organic substance to be measured.

The present invention will now be described in detail with reference to the accompanying drawings.

Fig. 1 is a schematical view showing a biofuel cell served as a microorganism dense-culture device in a BOD sensor of the present invention. Referring to Fig. 1, the device includes an anodic compartment 4 and a cathodic compartment 5. In these electrode compartments 4 and 5, there are defined an anode 1 and a cathode 2, respectively. Also, between the cathodic compartment 5 and the anodic compartment 4, there is interposed an ion exchange membrane 3 serving to divide these compartments from each other.

The cathodic compartment 5 is supplied with oxygen such that the cathode 2 is maintained at a potential different from the anode 1. The anodic compartment 4 is fed with a sample (such as wastewater and sludge) through a port 9, while the cathodic compartment 5 is fed with a phosphate buffer solution or tap water through a port 11. Also, the anodic compartment 4 is supplied with nitrogen through the port 9 such that it is maintained at an anaerobic condition. The cathodic compartment is supplied with air through a port 11 such that the electrodes 4 and 5 can be maintained at a potential different from each other. After a lapse of a certain amount of time (generally three weeks), on the anode 1, there is adhered electrochemically active microorganisms which were densely cultured using the wastewater as a substrate. The measurement of electricity generated from the oxidation of the substrate by the microorganisms allows an increase and decrease in BOD of the wastewater to be determined. In the present invention, the cathode 2 and the anode 1 are preferably made of a carbon felt, but these electrodes may be sometimes made of other materials. Further, a reference numeral 6 in Fig. 1 represents a leakage-preventing silicon rubber membrane, reference numerals 7 and 8 wirings of connecting the anode and the cathode, a reference numeral 10 a discharging port of a sample and nitrogen, the reference numeral 12 a discharging port of air and phosphate buffer solution, a reference numeral 13 a protective element, and a reference numeral 14 a fixing screw.

Fig. 4 is a schematical view showing a construction of a BOD sensor for carrying out an electrochemical dense-culture of a microorganism according to a preferred embodiment of the present invention. Referring to Fig. 4, the BOD sensor includes a potentiostat, in order for electrodes of the BOD sensor to be maintained at a constant voltage level. Moreover, in the BOD sensor, a working electrode 101 serves as an electron receptor and can be varied in electrochemical action to microorganisms, depending on a variation in applied voltage to the working electrode 101. The working electrode 101 is formed of a carbon felt, a reference electrode 113 is made of silver/silver chloride (Ag/AgCl), and an auxiliary electrode 102 is made of platinum. The reference electrode 113 serves to maintain and compensate the applied voltage to the working electrode 101. The auxiliary electrode 102 serves to constitute an electrical circuit along with the working electrode 101. The working electrode 101 is applied with a constant potential (generally, +0.98V with respect to the silver chloride reference electrode 113) and a working electrode compartment 104 is supplied with a sample (wastewater and sludge). Then, electrochemically active microorganisms are densely cultured for a certain time (generally, two weeks). As a result, the device shown in Fig. 4 having the microorganisms thus adhered (densely cultured) on the working electrode 101 can be used by itself as a BOD-measuring biosensor. Meanwhile, reference numerals 114 and 112 in Fig. 4 represent a magnetic stirrer and a check valve, respectively. Also, other reference numerals in Fig. 4 that were not described above will be described in Example 2 below.

The following examples are for further illustration purposes only and in no way limit the scope of this invention.

Example 1

Dense Culture of Electrochemically Active Microorganisms Using Biofuel Cell, and the Variation in Electric Current of Biofuel Cell according to COD

For the dense culture of electrochemically active microorganisms that utilize organic substances present in a certain wastewater as a substrate, a biofuel cell as shown in Fig. 1 was fabricated.

In this experiment, a wastewater from the starch processing (collected from Samyang Genex, Inchon, Korea) was used, and an active sludge generated from the wastewater treatment in the same factory was used as an inoculum. A basic configuration of the biofuel cell used in this example has referred to literature by Bennetto et al. [See, Bennetto et al., Biotechnology Letters, 7, 699-704, (1985)]. Referring to Fig. 1, both an anode 1 and a cathode 2 were formed of a carbon felt having a size of 5 x 7.5 x 0.6 cm, respectively. Also, the electrodes 1 and 2 were wired with a platinum wire. As used herein, the term "anodic compartment" designated by a reference numeral 4 means a place in which microorganisms or electron carriers of the microorganisms are oxidized by the anode 1. The term "cathodic compartment" designated by a reference numeral 5 means a portion in which electrons transferred through an external circuit reduce an oxidant in the cathode 2. The anodic compartment 4 and the cathodic compartment 5 were divided by an ion exchange membrane 3 from each other, and electrically connected through the external circuit. In this case, the connection of a suitable resistor to the external circuit allows a control of a flow of electric current between the cathode 2 and the anode 1. The cathodic compartment 5 (working capacity: 30 ml) was provided with air, while the anodic compartment 4 (working capacity: 30 ml) was added with a sample consisting of wastewater and sludge. After the sample had been added to the anodic compartment 4, the cathode 2 and the anode 1 were electrically connected via the resistor. Then, the anodic compartment 5 was supplied with nitrogen such that it was maintained at an anaerobic condition, whereas the cathodic compartment was supplied with air such that it was maintained at an aerobic condition. While maintaining these electrodes at the respective conditions, a dense culture of microorganisms was started. At about three weeks of the dense culture, a background current was maintained at a constant level. At this time, wastewater having a certain BOD value was added to the anodic compartment 4 and the total quantity of electric current being produced was integrated. When the generated electric current was indicated at a basic value, wastewater of another COD value (collected from Samyang Genex, Inchon, Korea) was added to the biofuel cell. As shown Fig. 2 in which arrows indicate COD values of the samples, the quantity of the generated electric current was increased in proportion to COD of

the added wastewater. Moreover, as shown in Fig. 3, the quantity of the generated electricity was increased in proportion to an increase in COD of the added sample.

Meanwhile, while operating the fabricated BOD measuring sensor for six months, a sample having 50 ppm of COD and a sample having 100 ppm of COD were added to the BOD sensor every one month, respectively, and the quantity of electricity being generated was measured. As shown in Fig. 10, the quantity of the generated electricity was maintained at a constant level with little or no change. As a result, it was confirmed that the BOD sensor could be operated while maintaining the quantity of the generated electricity at a constant level depending on the COD value of the added sample regardless of operation duration of the sensor.

Example 2

Dense Culture of Electrochemically Active Microorganisms using Biofuel Cell including Potentiostat, and the Variation in Electric Current according to COD

For an effective dense culture of an electrochemically active microorganism, a biosensor as shown in Fig. 4 was fabricated. Referring to Fig. 4, the biosensor includes an electrochemical cell 100 made of a pyrex glass and having a 500 ml capacity. At a portion of the electrochemical cell 100 in which a microorganisms will be densely cultured, a working electrode 101 made of a carbon felt is disposed while being connected to a potentiostat. Moreover, at another portion of the electrochemical cell 100, there is disposed an auxiliary electrode 102 made of a platinum wire to form an electrical circuit. A working electrode portion 104 having the working electrode 101 and an auxiliary electrode portion having the auxiliary electrode 102 are divided by a dialyzing diaphragm from each other. The working electrode portion 104 and the auxiliary electrode portion 105 were added with wastewaters having the same COD value. In order for the working electrode to be maintained at a constant potential, a reference electrode 113 was also disposed in the electrochemical cell 100. A potential of the working electrode 101 was adjusted by the potentiostat. Also, a port 109 for the introduction and discharge of the sample was formed on a side of the electrochemical cell 100. The electrochemical cell 100 was provided with nitrogen gas so that it was maintained at an anaerobic condition. For this purpose, a nitrogen introducing port 110 and a

nitrogen discharging port 111 are disposed that also may serve as the sample supplying and discharging ports when the sample needs to be continuously supplied. A variation in potential and current between the working electrode 101 and the auxiliary electrode 102 was amplified through the potentiostat and recorded with a recording unit using a computer and a recorder using a recording paper. For the dense culture of microorganisms, the working electrode portion 104 was added with an active sludge as an inoculum, and the potentiostat was then allowed to operate such that the working electrode 101 was maintained at a fixed potential. Thus, the dense culture of the microorganism was started. In this experiment, as the wastewater and active sludge, a wastewater from a starch processing (collected from Samyang Genex, Inchon, Korea) was used. The dense culture was started, after the wastewater and active sludge were added to the working electrode portion 104 and the working electrode 101 was fixed at +0.98V. At 14 days of operation after the experiment start, electric current between the working electrode 101 and the auxiliary electrode 102 was increased from about 50 μA to a maximum of 322 μA. At 18 days after the operation start, the electric current was stabilized at about 154 μA. When the electric current had been stabilized, the introduction of another wastewater of a different COD value through the sample introducing port 109 resulted in an increase in electric current value, similarly to that in Fig.2. While continuing to introduce and discharge wastewater through the sample introducing port 109 and the nitrogen discharging port 110, electric current between the working electrode 101 and the auxiliary electrode 102 was monitored. From this, it could be confirmed that the electric current was varied depending on COD of wastewater, as shown Fig. 5. Accordingly, it could be found that the use of the biosensor as in shown Fig. 4 permitted the continuous measurement of BOD. Moreover, observation of the electrode by a scanning electron microscope was carried out after the decomposition of the biosensor. From this observation, it could be confirmed that a large amount of microorganisms were adhered on the electrode, as shown in Figs. 6a and 6b that are micrographs of the electrode surface taken before and after the use, respectively. In addition, the microorganisms isolated from the electrode were cultured and then examined by a cyclic voltammetry. The microorganism was found to be electrochemically active.

Example 3

Change in Metal Salt-Reducing Bacteria Count Present in Anode and Anodic Compartment of Biofuel Cell Type BOD Sensor

A sample was collected from an anode and an anodic compartment during the dense culture and operation of a biofuel cell type BOD sensor used in Example 2, and was examined for a colony count of iron-reducing bacteria. In this experiment, a phosphate buffer solution-based medium (PBBM) was used as a medium. The following components were added to the medium to prepare a plate medium: 1g/L of an yeast extract, 1g/L of ammonium chloride, 25 ml/L of Macro-mineral (II) (including, per 1L, 6 g of KH_2PO_4, 12 g of NaCl, 2.4 g of $MgSO_4 \cdot 7H_2O$, and 1.6g of $CaCl_2 \cdot 2H_2O$), 2 ml/L of microelements (including 12.8 g of nitroacetic acid, 0.1 g of $FeSO_4 \cdot 7H_2O$, 0.1 g of $MnCl_2 \cdot 4H_2O$, 0.17 g of $CoCl_2 \cdot 6H_2O$, 0.1 g of $CaCl_2 \cdot 2H_2O$, 0.1 g of $ZnCl_2$, 0.02g of $CuCl_2 \cdot H_2O$, 0.1 g of H_3BO_3, 0.01g of molybdate, 1.0 g of NaCl, 0.017 g of $Na_2 \cdot SeO_3$, and 0.026 g of $NiSO_4 \cdot 6H_2O$), 0.1 ml/L of a vitamin solution (including 0.002 g of biotin, 0.002 g of folacin, 0.010 g of B6(pyridoxin)HCl, 0.005 g of B1(thiamin)HCl, 0.005 g of B2(riboflavin), 0.005 g of nicotinic acid(niacin), 0.005 g of panthothenic acid, 0.0001g of B12 (cyanocobalamine) crystal, 0.005 g of PABA, and 0.005 g of lipoic acid (thioctic acid)), 1ml/L of resazurin (0.2%), and 1.8% of agar agar.

As an electron donor, 20 mM of acetic acid, 30 mM of lactic acid, and 20 mM of glucose were used, respectively, while 20 mM of ferric pyrophosphate, a water soluble iron, was used as an electron receptor. In the first measurement, the respective samples of the aerobic sludge and the anaerobic sludge of the biofuel cell at the early stage of reaction were diluted with a physiological saline solution (0.85% brine) and then measured for Colony Forming Unit per ml of solution. Second and third measurements were carried out using the same medium and method as in the first measurement, at one month and two months after the reaction, respectively. Results are shown in Table 1 below.

Table 1: Change in Colony Count in Anodic Compartment of Biofuel Cell

Sample	Electron donor(mM)	Electron receptor(mM)	First time	Second time	Third time
Aerobic sludge	Acetic acid(20))	FP(20)	$2.8x10^{7}$	$0.9x10^{4}$	$5.1x10^{3}$
	Glucose(20)	FP(20)	$8.0x10^{7}$	$1.3x10^{5}$	$4.2x10^{4}$
	Lactic acid(30)	FP(30)	$6.4x10^{7}$	$1.1x10^{5}$	$4.1x10^{4}$
Anaerobic sludge	Acetic acid(20)	FP(20)	$3.6x10^{5}$	$5.4x10^{6}$	$1.5x10^{5}$
	Glucose(20)	FP(20)	$2.1x10^{5}$	$8.4x10^{6}$	$1.4x10^{6}$
	Lactic acid(30)	FP(20)	$1.7x10^{5}$	$1.5x10^{6}$	$2.3x10^{5}$

FP: Ferric Pyrophosphate

As evident from Table 1 above, in the case of the aerobic sludge sample, it is believed that, as the anodic compartment of the biofuel cell is maintained in an anaerobic condition, strains other than facultative anaerobic strains are continued to reduce while being screened, such that only electrochemically active microorganisms are densely cultured. In the case of the anaerobic sludge sample, the anaerobic bacteria were increased at the second measurement, and then decreased at the third measurement, such that only electrochemically active microorganisms were densely cultured.

Example 4

Measurement of Lactic Acid Concentration with Fuelcell Type Biosensor Using *Shewanella putrefaciens*

For the measurement of lactic acid concentration, a biosensor as shown in Fig. 7 was fabricated using *Shewanella putrefaciens* IR-1, a kind of an iron-reducing bacterium. Such a strain can be available from the Korean Collection for Type Cultures, Korean Research Institute of Bioscience and Biotechnology, under the accession number KCTC 8753P. This bacterium has an ability to reduce ferric oxide using a reducing power generated in the oxidation of lactic acid into acetic acid.

Referring to Fig. 7, the biosensor includes a cell 200 in which an anodic compartment 204 and a cathodic compartment 205 are defined. The anodic

compartment 204 and the cathodic compartment 205 are divided by a cation exchange membrane 203 and include an anode 201 and a cathode 202, respectively. The cathodic compartment 205 having a 20 ml capacity was charged with 0.05 M of a phosphate buffer solution containing 0.1 M of sodium chloride. An anodic compartment 204 was fed with nitrogen through a nitrogen-introducing port 211. A reference numeral 210 represents a nitrogen-discharging port. Also, the anodic compartment 204 was added with *Shewanella putrefaciens* IR-1 (dry weight: 5 mg) and 19 ml of a 0.05 M phosphate buffer solution containing 0.01 M sodium chloride. The anode 201 was made of a carbon felt having a size of 0.8 cm x 4 cm x 0.3 cm, and a cathode 202 was made of a reticulated vitreous carbon having a size of 3 cm x 3 cm x 0.3 cm. The anode 201 and the cathode 202 were electrically connected with each other via a resistor (500 Ω). In this state, a variation in voltage across the resistor was measured with a voltage-measuring unit, and converted into electric current between the two electrodes. Electric current was amplified through a scanner so that a recording unit could be operated. The recording unit has recorded a variation in electric current (voltage). Working temperature was maintained at 25 °C. After background current was stabilized, 1 ml of the respective samples containing lactic acid at a different concentration were fed into the biofuel cell through a sample-introducing port 209. A variation in electric current according to time was recorded, and an initial slope of electric current was obtained.

The initial slope of electric current generated when introducing lactic acid of a desired concentration into the biosensor was proportional to a lactic acid concentration. This indicates that electrons generated from the oxidation of lactic acid by the microorganism move toward the electrode and that the lactic acid concentration is proportional to the quantity of electrons generated at a constant microorganism concentration. Fig. 8 illustrates the typical increase in electric current according to the lactic acid addition, and Fig. 9 illustrates the initial slope of electric current according to a variation in lactic acid concentration. A correlation coefficient of the initial current slope with the lactic acid concentration was 0.84. This improvement in correlation coefficient was obtained by changing the biosensor construction, such as the nature and concentration of the microorganism, the material and size of the electrodes, the resistor and the like.

Industrial Applicability

As apparent from the foregoing, the biosensor of the present invention utilizes electrochemically active bacteria that were contained in wastewater and sludge and densely cultured during the operation procedure of the biofuel cell for the BOD measurement, as a microbial catalyst of the biofuel cell used in the biosensor. Therefore, the present biosensor can be operated without the artificial addition of microorganisms, and allows an activity of the bacteria to be maintained at a suitable level depending on the nature of wastewater. Moreover, it enables the continuous measurement for the BOD value of wastewater. In addition, the biofuel cell used in the BOD-measuring biosensor of the present invention can be operated in a stable manner over six months or more.

Although the preferred embodiments of the invention have been disclosed for illustrative purposes, those skilled in the art will appreciate that various modifications, additions and substitutions are possible, without departing from the scope and spirit of the invention as disclosed in the accompanying claims.

Claims

1. A BOD-measuring biosensor, comprising a measuring unit, an electric current-detecting unit, and a recording unit serving to record a variation in the detected electric current, the measuring unit being composed of a mediator-less biofuel cell, the biofuel cell including:

cathodic and anodic compartments defined therein and contained with a conductive medium, respectively;

an anode arranged in the anodic compartment;

a cathode arranged in the cathodic compartment; and

an ion exchange membrane interposed between the cathodic and anodic compartments and serving to divide the anodic compartment from the cathodic compartment, wherein the anodic compartment is fed with a sample containing electrochemically active bacteria.

2. The BOD-measuring biosensor of Claim 1, in which the measuring unit further comprises a potentiostat serving to control a potential of the anodic compartment.

3. A method for measuring BOD of a sample using the BOD-measuring biosensor according to Claim 1 or 2, the method comprising:

electrically connecting the anode to the cathode via a resistor;

introducing the anodic compartment with nitrogen to maintain in an anaerobic condition, while introducing the cathodic compartment with oxygen to maintain in an aerobic condition;

densely culturing an electrochemically active bacterium present in the sample in the anodic compartment; and

measuring electric current being generated while employing the densely cultured, electrochemically active bacteria as a microbial catalyst.

4. A mediator-less biofuel cell type biosensor for the measurement of organic substance concentration, the biosensor comprising a measuring unit, an

electric current-detecting unit, and a recording unit serving to record a variation in the detected electric current, the measuring unit comprising:

cathodic and anodic compartments defined therein and contained with a conductive medium, respectively;

an anode arranged in the anodic compartment;

a cathode arranged in the cathodic compartment; and

an ion exchange membrane interposed between the cathodic and anodic compartments and serving to divide the anodic compartment from the cathodic compartment, wherein the anodic compartment contains a sample containing a single species of an electrochemically active bacterium serving to catabolize a desired organic substance.

5. A method for measuring a concentration of an organic substance using the biosensor according to Claim 4, the method comprising:

adding a sample to be measured to the anodic compartment while continuing to feed air to the cathodic compartment to maintain the cathodic compartment at a voltage different from the anodic compartment; and

measuring an electric current generated from the consumption of an organic substance contained in the sample by the electrochemically active bacterium, whereby the concentration of the organic substance is measured.

6. A method for densely culturing electrochemically active bacteria present in active sludge and wastewater, using the mediator-less biofuel cell included in the biosensor according to Claim 4, the method comprising:

adding the active sludge and the wastewater to the anodic compartment;

electrically connecting the anodic compartment to the cathodic compartment via a resistor;

introducing nitrogen to the anodic compartment to maintain the anodic compartment in an anaerobic condition, while introducing air to the cathodic compartment to maintain the cathodic compartment in an aerobic condition; whereby a bacterium present in the active sludge and wastewater is densely cultured without a separate electron receptor.

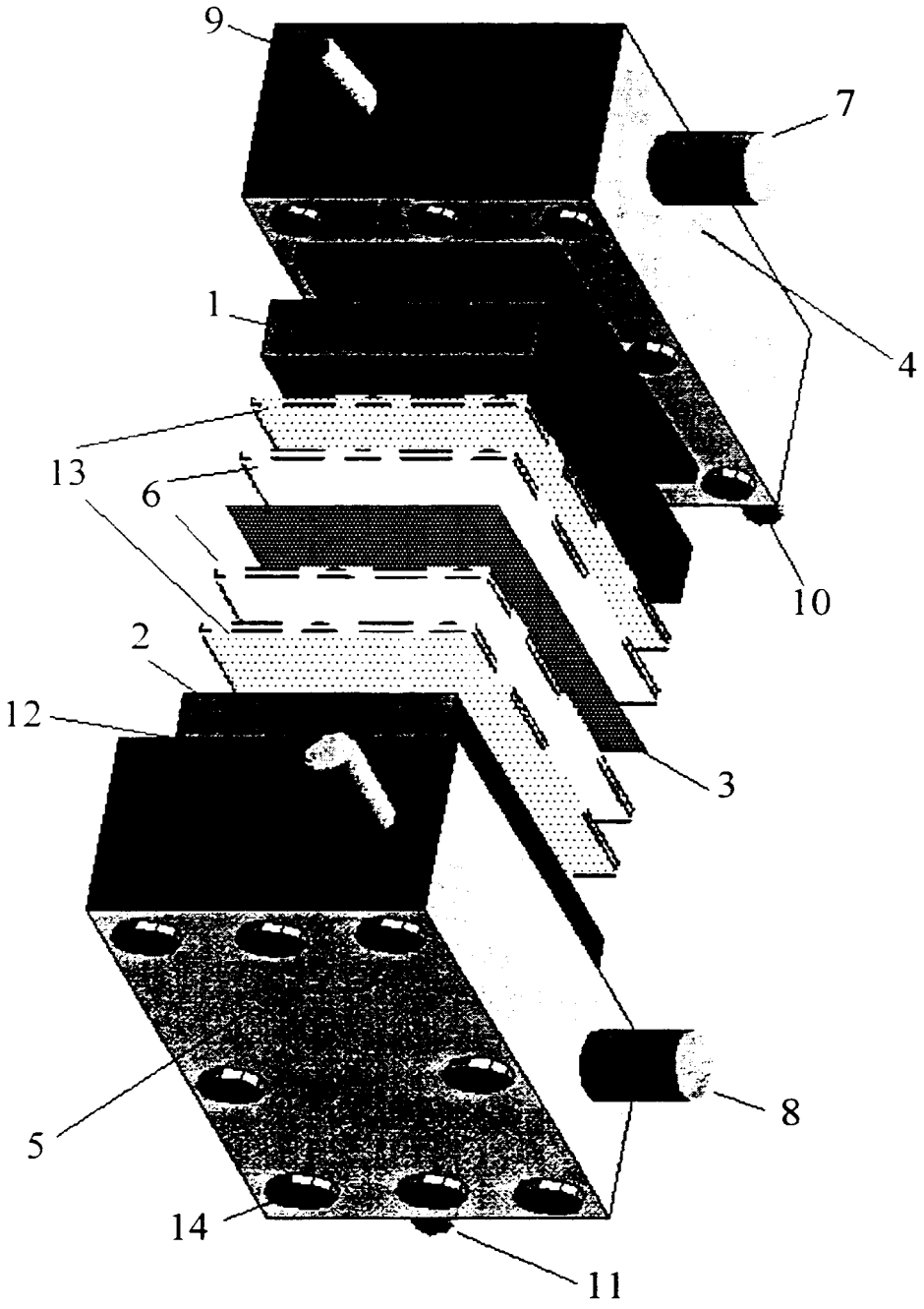

Fig. 1

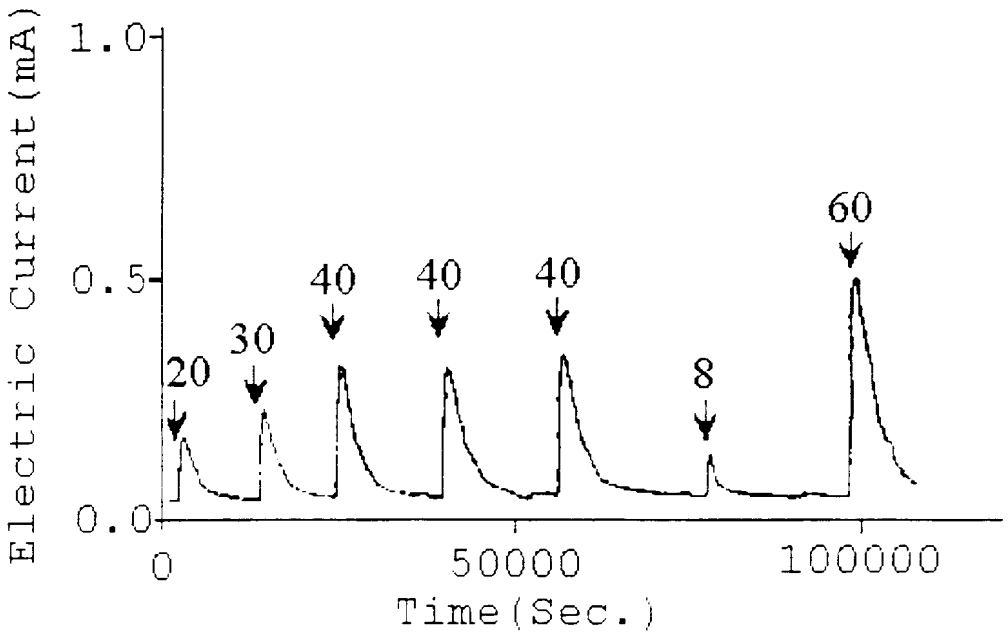
Electric Current(mA)
1.0
0.5
0.0
0
50000
100000
Time(Sec.)
20
30
40
40
40
8
60

Fig. 2

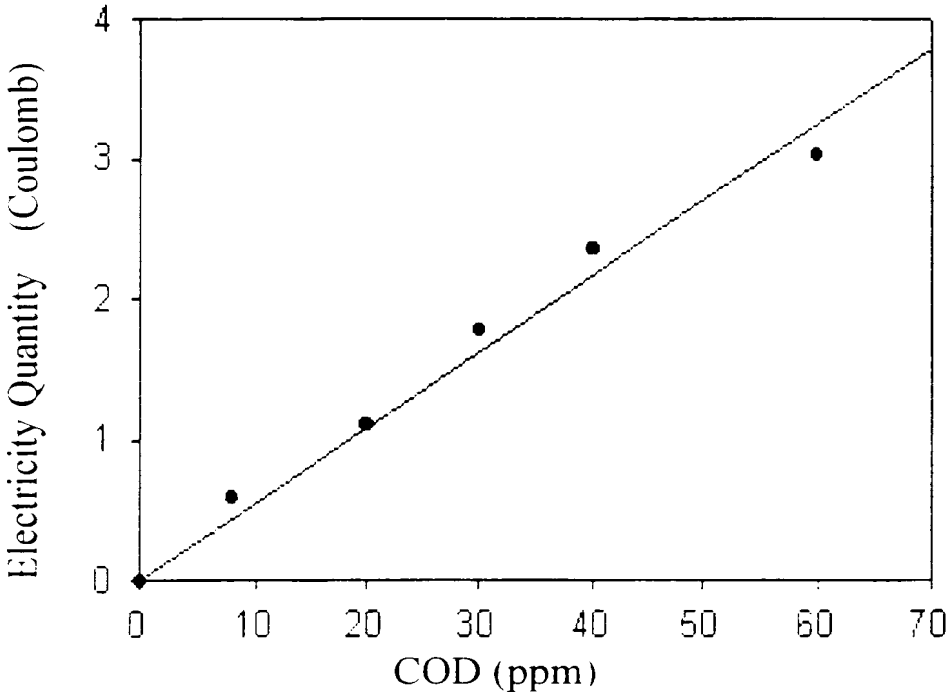
Electricity Quantity (Coulomb)
4
3
2
1
0
0
10
20
30
40
50
60
70
COD (ppm)

Fig. 3

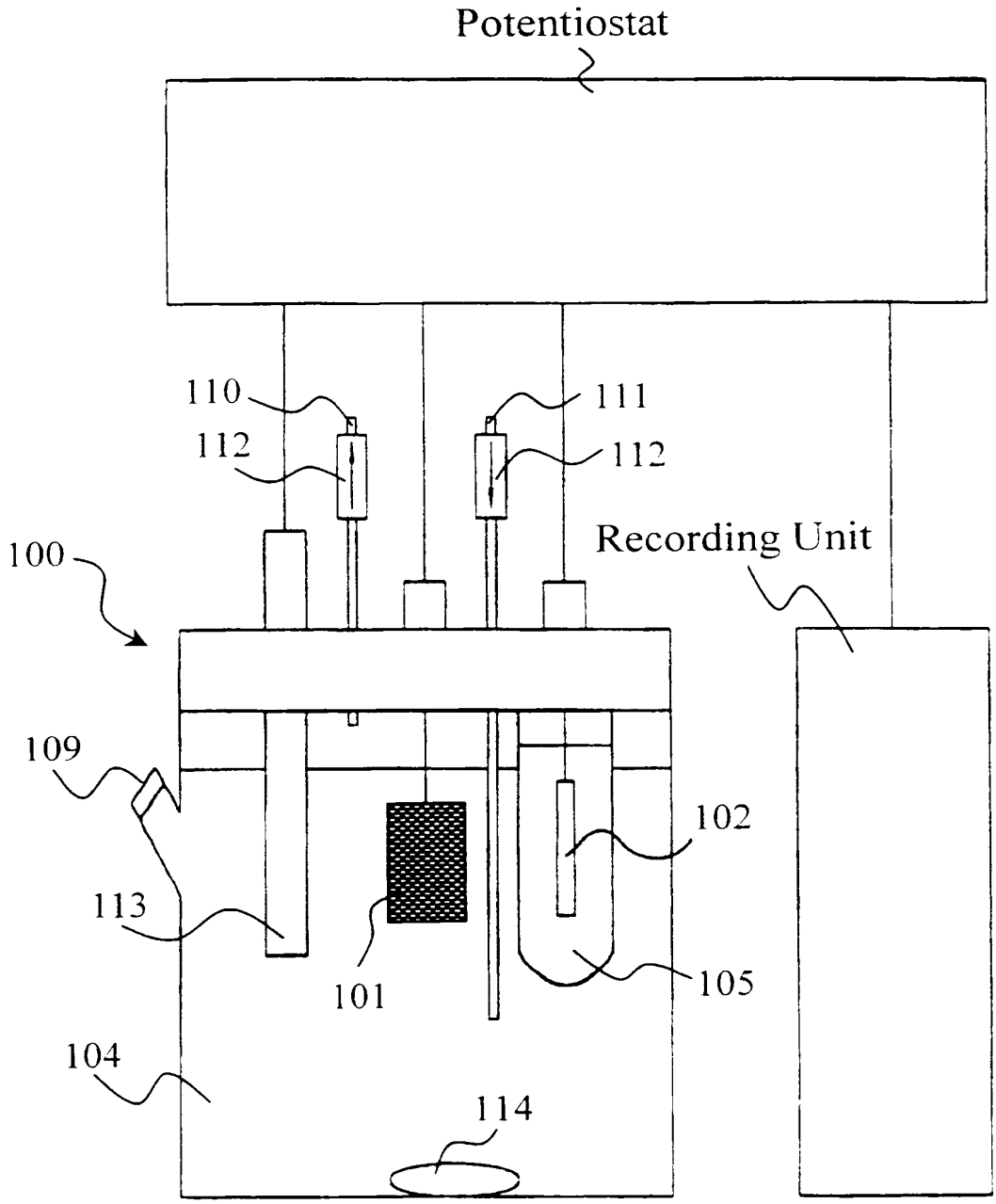
Potentiostat
110
111
112
112
Recording Unit
100
109
102
113
101
105
104
114

Fig. 4

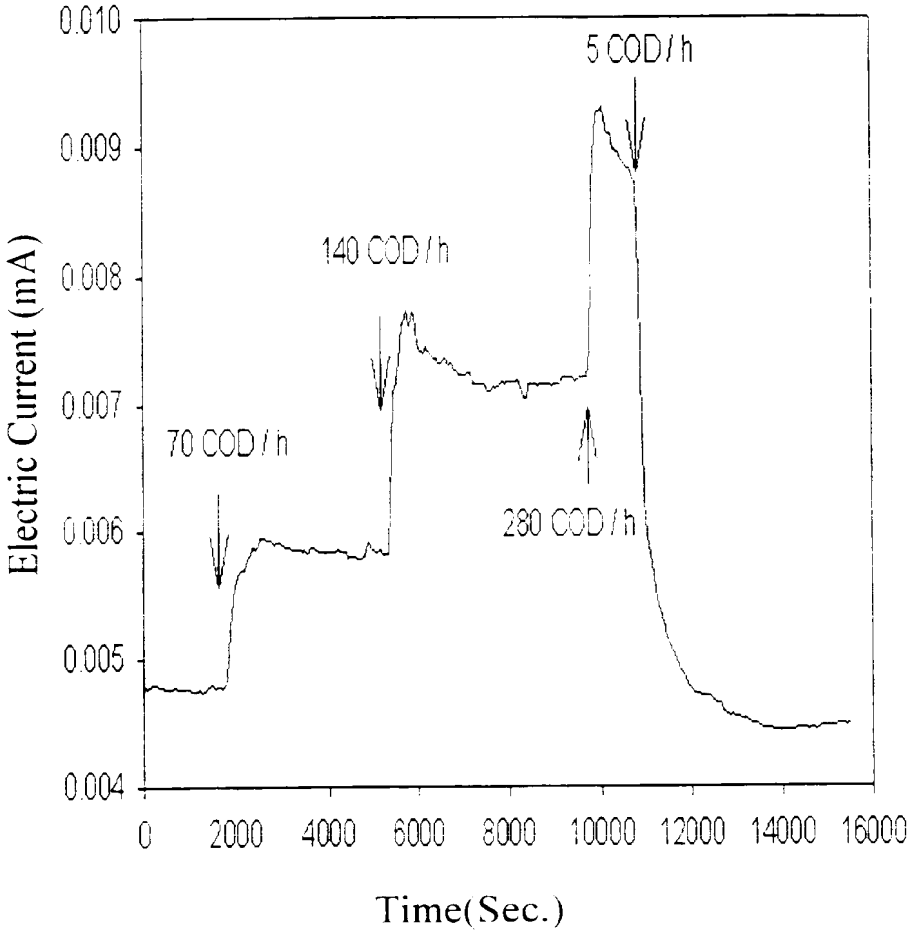
0.010
0.009
0.008
0.007
0.006
0.005
0.004
Electric Current (mA)
5 COD / h
140 COD / h
70 COD / h
280 COD / h
0
2000
4000
6000
8000
10000
12000
14000
16000
Time(Sec.)

Fig. 5

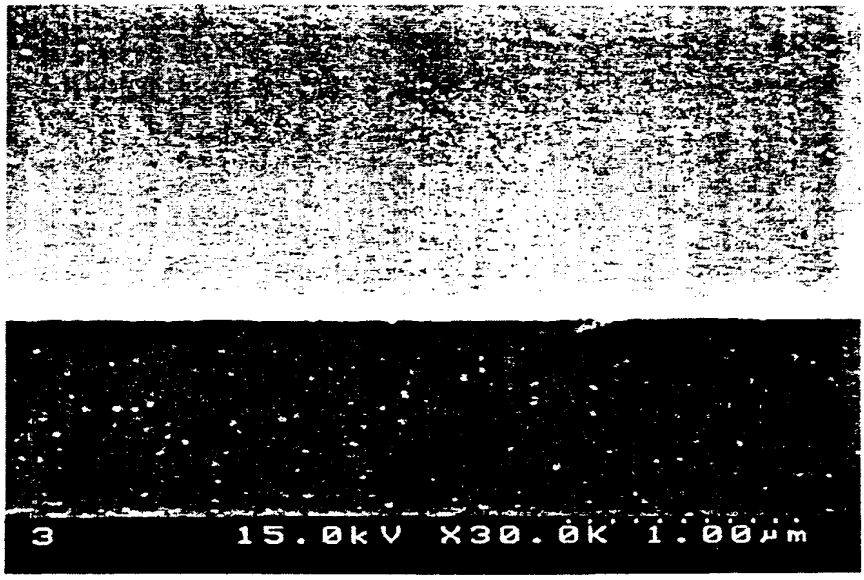

Fig. 6a

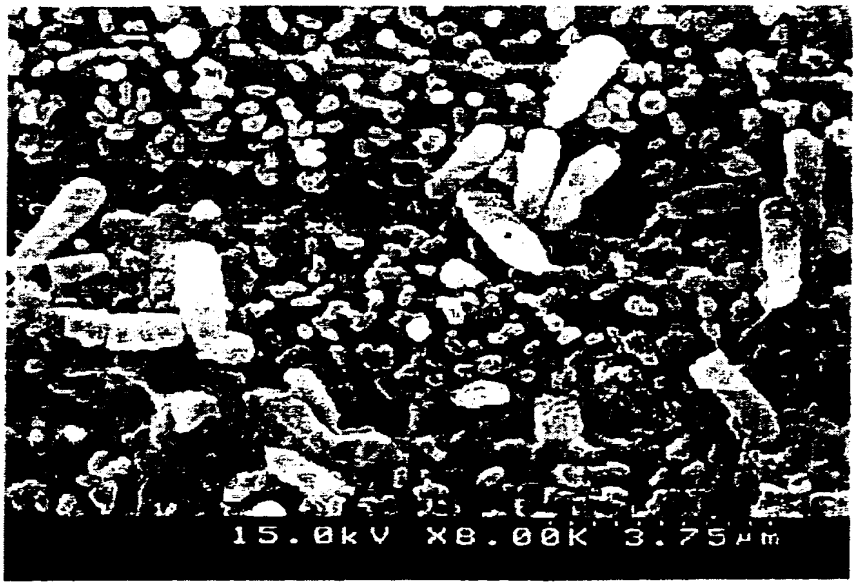

Fig. 6b

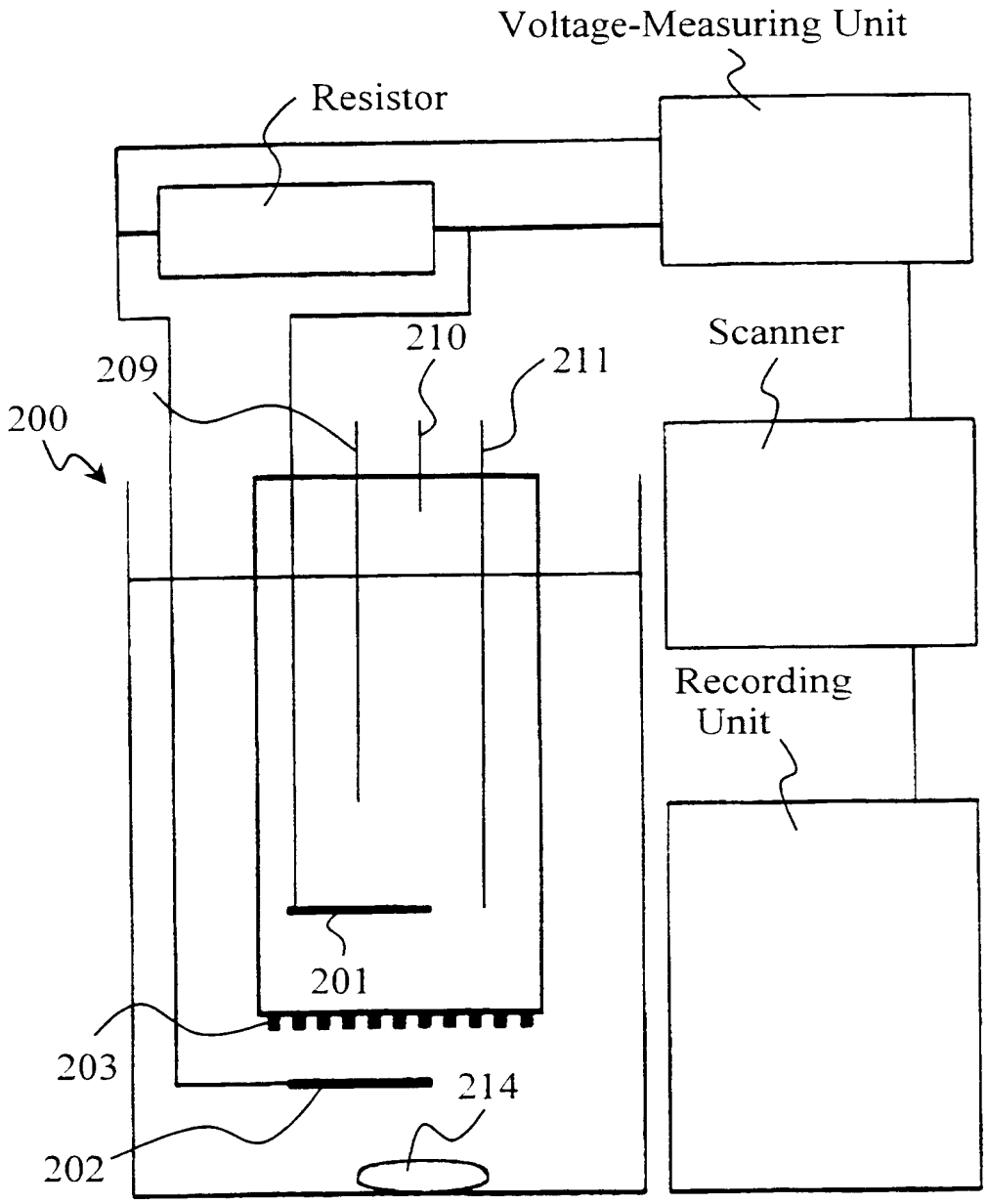
Voltage-Measuring Unit
Resistor
Scanner
210
211
209
200
Recording
Unit
201
203
214
202

Fig. 7

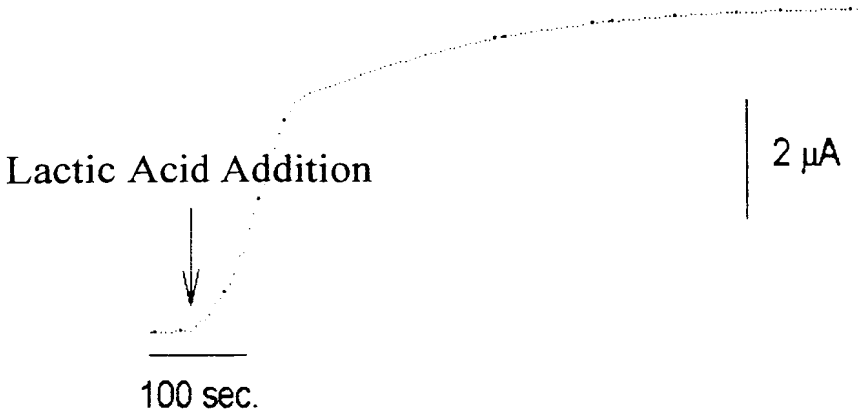
Lactic Acid Addition
2 μA
100 sec.

Fig. 8

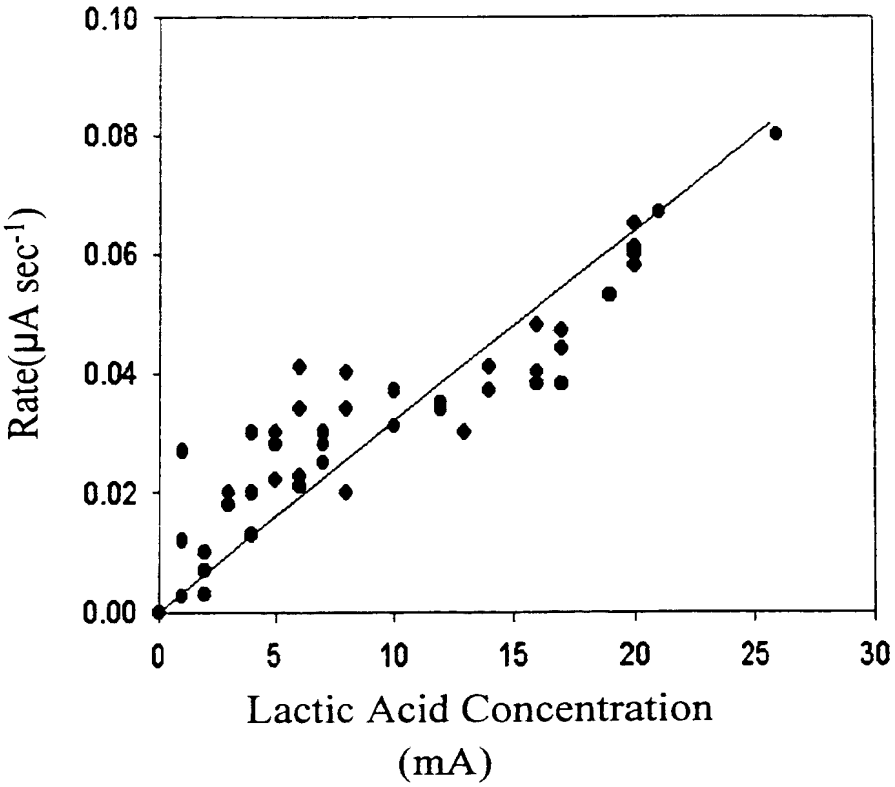
0.10
0.08
0.06
0.04
0.02
0.00
Rate(μA sec-1)
0
5
10
15
20
25
30
Lactic Acid Concentration
(mA)

Fig. 9

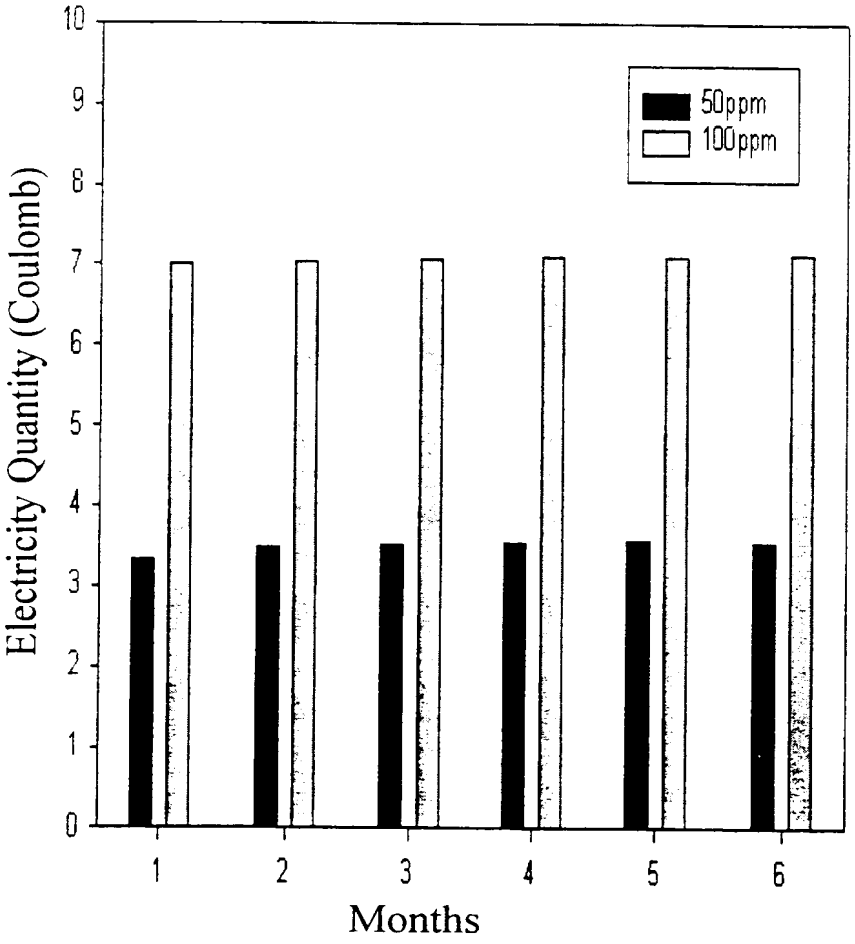
Electricity Quantity (Coulomb)
0
1
2
3
4
5
6
7
8
9
10
50ppm
100ppm
1
2
3
4
5
6
Months

Fig. 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR00/00230

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 G01N 33/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimun documentation searched (classification system followed by classification symbols)

IPC7 G01N 27/26, G01N 27/327, C01N 27/416,H01M 8/16

Documentation searched other than minimun documentation to the extent that such documents are included in the fileds searched

Korean patents and applications for inventions since 1975
Korean Utility models and applications for Utility models since 1975
Japanese Utility models and applications for Utility models since 1975

Electronic data base consulted during the internationl search (name of data base and, where practicable, search trerms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A	KR 98-016777 A (Korea Institute of Science and Techology) 05 JUNE 1998 - Claim 1-5, Figure1-	1-6
A	JP 10-318965 A (FUJI ELECTRIC) 04 DECEMBER 1998 - See the whole document -	1-6
A	JP 07-35741 A (FUJI ELECTRIC) 07 FEBRUARY 1995 - See the whole document -	1-6
P, A	KR 99-69205 A (KYU-SUNG LEE) 06 SEPTEMBER 1999 - Claim 1-4, Figure1-	1-6

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevence
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevence; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevence; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 JUNE 2000 (27.06.2000)	29 JUNE 2000 (29.06.2000)

Name and mailing address of the ISA/KR	Authorized officer
Korean Industrial Property Office Government Complex-Taejon, Dunsan-dong, So-ku, Taejon Metropolitan City 302-701, Republic of Korea	JOO, Young Sik
Facsimile No. 82-42-472-7140	Telephone No. 82-42-481-5995

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/KR00/00230

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
KR 98-016777 A	05. 06. 1998	NONE	
JP 10-318965 A	04. 12. 1998	NONE	
JP 07-035741 A	07. 02. 1995	NONE	
KR 99-06920 A	06. 09. 1999	NONE	

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PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

6

Applicant's or agent's file reference 2000-P-34	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT IPEA 416)
International application No. **PCT/KR00/00230**	International filing date *(day month year)* 17 MARCH 2000 (17.03.2000)	Priority date *(day month year)* 07 JULY 1999 (07.07.1999)

International Patent Classification (IPC) or national classification and IPC

IPC7 G01N 33/18

Applicant

Korea Institute of Science and Technology et al

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of _____ sheets.

3. This report contains indications relating to the following items:

- I [X] Basis of the report
- II [ ] Priority
- III [ ] Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV [ ] Lack of unity of invention
- V [X] Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI [X] Certain documents cited
- VII [ ] Certain defects in the international application
- VIII [ ] Certain observations on the international application

Date of submission of the demamd	Date of completion of this report
02 FEBRUARY 2001 (02.02.2001)	27 OCTOBER 2001 (27.10.2001)

Name and mailing address of the IPEA KR	Authorized officer
Korean Intellectual Property Office Government Complex-Daejeon, Dunsan-dong, Seo-gu, Daejeon Metropolitan City 302-701, Republic of Korea	MIN, Man Ho
Facsimile No. 82-42-472-7140	Telephone No. 82-42-481-5578

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International aplication No
PCT KR00/00230

I Basis of the report

1. With regard to the elements of the international application *

[X] the international application as originally filed

[ ] the description:
pages _____ . as originally filed
pages _____ . filed with the demand
pages _____ . filed with the letter of _____

[ ] the claims:
pages _____ . as originally filed
pages _____ . as amended (together with any statment) under Article 19
pages _____ . filed with the demand
pages _____ . filed with the letter of _____

[ ] the drawings:
pages _____ . as originally filed
pages _____ . filed with the demand
pages _____ . filed with the letter of _____

[ ] the sequence listing part of the description:
pages _____ . as originally filed
pages _____ . filed with the demand
pages _____ . filed with the letter of _____

2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language English which is

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b))

[X] the language of publication of the international application(under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination(under Rules 55.2 and or 55.3).

3. With regard to any **nucleotide** and or amino acid sequence disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

[ ] contained inthe international application in written form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international applicationas as filed has been furnished

[ ] The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

4. [ ] The amendments have resulted in the cancellation of:

[ ] the description, pages _____

[ ] the claims, Nos. _____

[ ] the drawings, sheet _____

5. [ ] This opinion has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box(Rule 70.2(c)) **

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

** *Any replacement sheet containing such amendments must be referred to under item I and annexed to this report.*

INTERNATIONAL [redacted]ELIMINARY EXAMINATION

[redacted]ernational aplication No
PCT KR00 00230

V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-6	YES
	Claims		NO
Inventive step (IS)	Claims	1-6	YES
	Claims		NO
Industrial applicability (IA)	Claims	1-6	YES
	Claims		NO

2. Citations and explanations (Rule 70.7)

The present application pertains to a bio-sensor for the measurement of an organic substance concentration and BOD.

The biosensor utilizes an organic substance-magnetizing force and electron transfer capacity of electrochemically active microorganisms without an electron transfer mediator or a transducer.

The international Search Report revealed four documents of interest:

A) KR98-016777

B) JP10-318965

C) JP07-35741

D) KR99-69205

Document A which is cited as a reference in the present application discloses a biofuel cell using a metal salt-reducing bacteria. Document B relates to the BOD measuring method by suppressing the deterioration of the standard solution. Document C discloses a bio-sensor measuring BOD using a microorganism eating organic substances combined with a bio-sensor measuring N-BOD(BOD based on nitrification) using a bacterium eating ammonia nitrogen. Document D concerns a bio-sensor for measuring BOD employing immobilized *Peudomonas*.

These cited documents in the Search Report show the general state of the art. None of these documents reveal a biosensor using densely cultured electrochemically active bacteria contained in wastewater and sludge as a microbial catalyst.

Therefore, claims 1-6 are considered to fulfil the requirements of novelty, inventive step and industrial applicability.

INTERNATIONAL PRELIMINARY EXAMINATION

ernational aplication No
PCT KR00 00230

VI Certain documents cited

1 Certain published documents (Rule 70.10)

Application No Patent No	Publication date *(day month year)*	Filing date *(day month year)*	Priority date (valid claim) *(day month year)*
P.A. KR99-06920	06 September 1999	05 February 1998	05 February 1998

2. Non-written disclosures (Rule 70.9)

Kind of non-written disclosure	Date of non-written disclosure *(day month year)*	Date of written disclosure referring to non-written disclosure *(day month year)*